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Identification of Yeast Deubiquitinating Enzyme (DUB) Substrates

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IDENTIFICATION OF YEAST DEUBIQUITINATING ENZYME (DUB) SUBSTRATES

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ABSTRACT

E3 ubiquitin ligases, such as SCF^{Grr1}, are enzymes that add ubiquitin chains to proteins targeting them to the proteasome for degradation. Deubiquitinating enzymes (DUBs) can counteract this activity by removing ubiquitin chains and thus rescue proteins from degradation. Our goal was to develop genetic and biochemical screening approaches to identify DUB substrates, and thus learn more about DUBs that may contribute to human disease. Our data suggests that the yeast DUBs Ubp3 and Ubp12 affect the stability of the Grr1 targets Cln2, Cln3, and Gic2.

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BACKGROUND

The Cell Cycle

In order for eukaryotic cells to successfully replicate and divide, they go through a unidirectional process called the cell cycle (**Figure-1**). The cell cycle consists of four phases: G1, S, G2, and M. Together, G1, S, and G2 phases are called interphase because it is during this time that preparations for cell division occur. During G1 phase, cells grow and commit to entering the cell cycle. G1 is followed by the S phase (DNA synthesis phase), when DNA replication occurs, then enter G2, a second growth phase when the duplicated chromosomes are checked to make sure they were copied properly. Finally, during M phase, or mitosis, duplicated chromosomes are segregated to daughter cells and cell division (cytokinesis) occurs (Cooper, 2000). Mitosis can be further subdivided into prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. During these stages, DNA condenses and the nuclear membrane breaks apart, chromosomes line up at the equator of the spindles, spindle fibers pull sister chromatids to opposite poles of the cell, and the cell pinches in half to form two new cells while the nucleus reforms around the DNA (Carter, 2010).

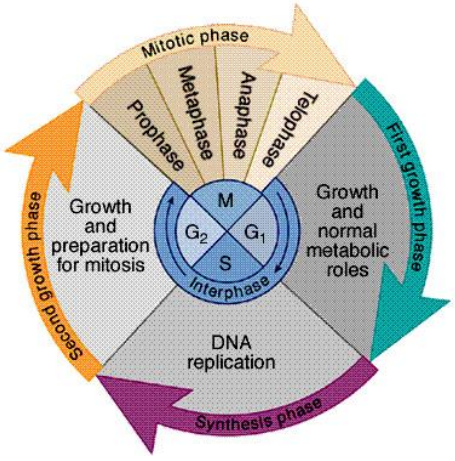

Figure-1: The Cell Cycle. (Anderson, 2010)

Cell Cycle Checkpoints

A cell must complete the full cell cycle in order to divide properly. Therefore, in order to make sure that a cell is prepared to divide, there are various checkpoints throughout the cycle to ensure that no errors were made before the cell progresses into the next phase. In mammalian cells, checkpoints control the activity of cyclin-dependent kinases (Cdks), whose activities are required for the cell cycle to proceed (Johnson & Walker, 1999). For example, a G2 phase checkpoint is activated if DNA damage is found after S phase. If this occurs, the cell cycle is halted through the inhibition of Cdk1-cyclin B complex, which prevents the cell from entering M phase (Lindqvist, 2009). If the damage can be fixed, then it will be, and the cell will segregate its chromosomes and divide. But, if the damage is unable to be repaired, then the cell will arrest or die, in a process known as apoptosis.

Cancer is a disease caused by uncontrolled cell division. Therefore, misregulation of the cell cycle can lead to cancer (Cohen & Tcherpakov, 2010; Cristina, 2011). If checkpoints are not functioning properly, then a cell with DNA mutations may pass through the cycle and divide, potentially leading to uncontrolled cell division and tumor formation. The most commonly mutated protein in cancer cells is the tumor suppressor p53, which normally functions to arrest cells in G1 and/or G2 phase following DNA damage (Nigro, 1989). If p53 is mutated, then cells will continue to divide even though DNA damage is present, and this can lead to the development of a tumor. Therefore, understanding the proteins and mechanisms that control the cell cycle will aid in our understanding of how cancer develops, and allow us to develop better ways to treat the disease.

The Importance of Regulating Protein Levels Throughout the Cell Cycle

Fluctuations in cell cycle protein levels drive cell cycle progression, and this occurs through a combination of the cyclical transcription of cell cycle genes and degradation of the corresponding proteins. A large fraction of the genome is expressed in this cyclical way; for example, one-sixth of the yeast genome is transcribed in a cell cycle-dependent manner (Spellman, et al., 1998). The degradation of previously expressed proteins, and the expression of new proteins, helps ensure that the cell continues the cycle and does not go backwards (King & Cidlowski, 1998). Cell cycle proteins that are transcribed at high levels at the beginning of a specific phase are often degraded rapidly near the end of that phase. For example, cyclin E is rapidly synthesized in late G1, and rapidly degraded in mid-S (**Figure-2**). This regulation ensures that DNA does not get re-replicated before the next cell cycle, which can lead to mutations in the genome. Therefore, the properly timed expression and degradation of these cell cycle control proteins is essential for a complete and accurate cell cycle.

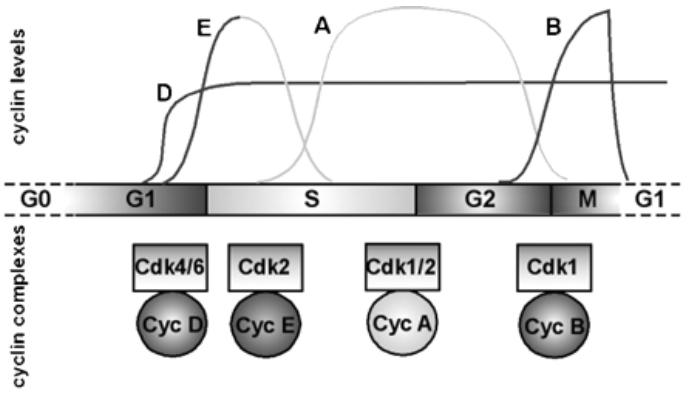

Figure-2: Fluctuation in Cyclin Levels Throughout the Cell Cycle. (Verschuren, 2004)

Protein Degradation

Degradation of cellular proteins occurs by two separate mechanisms: non-specific degradation and regulated degradation. Non-specific degradation occurs by autophagy in the mammalian lysosome or yeast vacuole, and leads to the bulk turnover of cytoplasmic material. In this process, cellular components are engulfed non-selectively in vesicles called "autophagic bodies" and then brought to the vacuole where the vesicle is broken down then all the contents inside are degraded to allow for cellular components to be recycled (Kim & Klionsky, 2000). Autophagy typically occurs when the cell is starved and needs to obtain more nutrients.

Regulated degradation occurs through the ubiquitin-proteasome pathway, in which specific proteins are marked for degradation by the addition of ubiquitin chains that target them to the proteasome. Many cell cycle proteins are regulated through this ubiquitin-proteasome degradation pathway (**Figure-3**). The energy of ATP is used to form a thiol ester bond between ubiquitin and an activating enzyme E1 (Ventii & Wilkinson, 2008). The activated ubiquitin is then attached to one of many E2s (ubiquitin-conjugating enzymes), and then transferred to the appropriate protein target by an E3 ubiquitin-ligase forming an isopeptide bond (Hershko & Ciechanover, 1998).

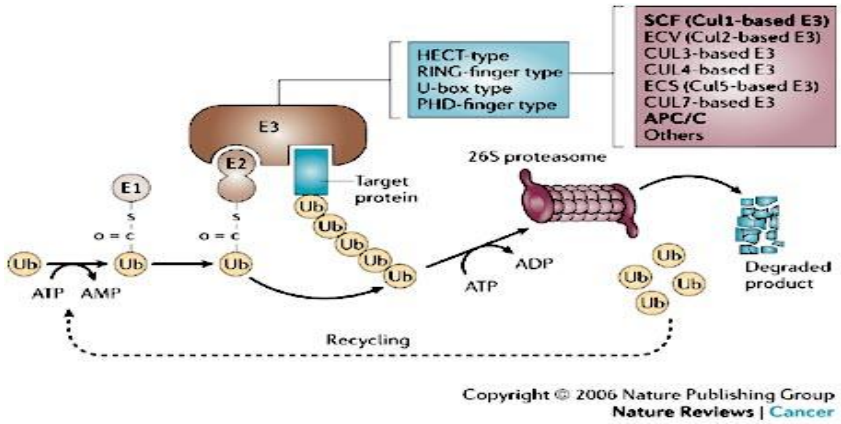

Figure-3: Proteolysis Using Ubiquitin. (Nakayama 2006)

The E3 ubiquitin ligase continues to add ubiquitins to the target using the 7 lysine residues of ubiquitin as attachment sites to form ubiquitin chains. Therefore, multiple chain structures can be made, and different chain linkages determine different target outcomes (**Figure-4**). Only K48- and K11-linked chains (as listed in example B in the figure), with a minimum of four ubiquitins, signals the target to the 26S proteasome where it is degraded into short peptides (Coux, et al., 1996; Nijman, et al., 2005).

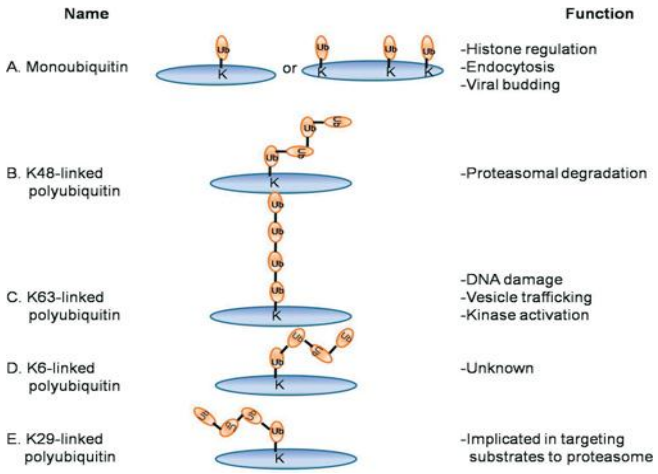

Figure-4: Ubiquitin Chain Signals. (Ventii & Wilkinson, 2008)

E3 Ubiquitin Ligases

The functions of most E3s that exist in eukaryotic cells are unknown. There are two families of E3s, those containing HECT domains and those with RING finger domains (Chen, et al., 2006). RING ligases act by bringing together E2-ubiquitin conjugates and the protein target to catalyze the transfer of ubiquitin, while HECT E3s become conjugated to the ubiquitin before transferring to the target. RING domain E3s can be further subdivided into monomeric RINGs and multi-subunit RINGs, which function as large protein complexes. The two best-characterized multi-subunit RING E3s are the Anaphase Promoting Complex/Cyclosome (APC/C) and Skp1-

Cullin-F-box Complexes (SCF) (Petroski & Deshaies, 2005) (**Figure-3**). The APC is mainly active during mitosis and G1 phases. Its essential functions are to degrade mitotic cyclins and ubiquitinate securin, an anaphase inhibitor, allowing for sister chromatid separation. SCF complexes are present throughout the cell cycle, evolutionary conserved, and have more diverse functions than the APC. As the name suggests, SCF complexes consist of several components: the adaptor protein Skp1, a structural cullin subunit (Cul1 in mammals, Cdc53 in yeast), and the RING finger protein Rbx1 (Deshaies, 1999). In addition, each SCF E3 contains one of a large family of F-box proteins that bind to specific substrates, usually in a phosphorylation-dependent manner. One of the best-characterized roles of SCF E3s is to ubiquitinate cell cycle regulatory proteins, such as cyclins and cdk inhibitors, through the specific interaction of the F-box protein with the phosphorylated targets (Vodermaier, 2004).

Ubiquitin Ligases in Budding Yeast

The budding yeast, *Saccharomyces cerevisiae*, is used as a model organism to study complex eukaryotic processes such as cell cycle regulation for several reasons. Its genome is translatable to humans (Bentley & Carr, 1997; Freire, et al., 1998; Sanchez, et al., 1997), but the yeast genome is less complex than the human genome, consisting of 16 linear chromosomes and approximately 6000 genes (Zagulski, et al., 1998). In addition, the cell cycle phases can be visibly observed in yeast based on cell shape, making cell cycle progression easy to monitor. Yeast is also easy to maintain, easy to genetically manipulate, and have a generation time of only 90 minutes (Perego, et al., 2000).

The SCF ubiquitin ligase containing the F-box protein Grr1 (SCF^{Grr1}) is one of the best-characterized E3s in budding yeast. Cells lacking *GRR1* show several abnormalities, such as elongated cell morphology, loss of glucose repression, and filamentous defects (Blacketer, et al.,

1995; Flick & Johnston, 1991; Loeb, et al., 1999). Moreover, SCF^{Grr1} plays an important role in regulating the cell cycle because it targets all three G1 cyclins: Cln1, Cln2, and Cln3 (Barral, et al., 1995; Benanti et al, submitted) (**Figure-5**).

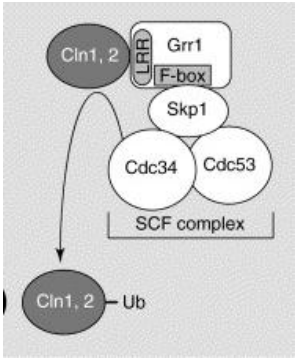

Figure-5: Ubiquitination of Cln1 and Cln2 Through the Grr1 SCF Complex. (Johnston, 1999)

Other known Grr1 targets include the Cdc42 GTPase-associated protein Gic2 (Jaquenoud, et al., 1998), which is involved in bud initiation and polarizing actin cytoskeleton, Hof1, which helps to create a functional actomyosin ring early in mitosis and complete cytokinesis (Blondel, et al., 2005), and the glycolysis regulators Tye7 and Pfk27 (Benanti, et al., 2007). Since the pathways regulating the degradation of Grr1 targets are well-understood, these proteins serve as good model substrates to study regulation of the ubiquitin-proteasome system.

Deubiquitinating Enzymes

The action of ubiquitin ligases can be further regulated by deubiquitinating enzymes (DUBs) that function to remove or remodel ubiquitin chains (Ventii & Wilkinson, 2008). Additionally, it is important to learn about the function of DUBs because they are often found mutated in many diseases, including cancer (Hussain, et al., 2009). DUBs have several

regulatory roles in the ubiquitin pathways (**Figure-6**). For instance, they can remove ubiquitin chains from proteins saving them from degradation.

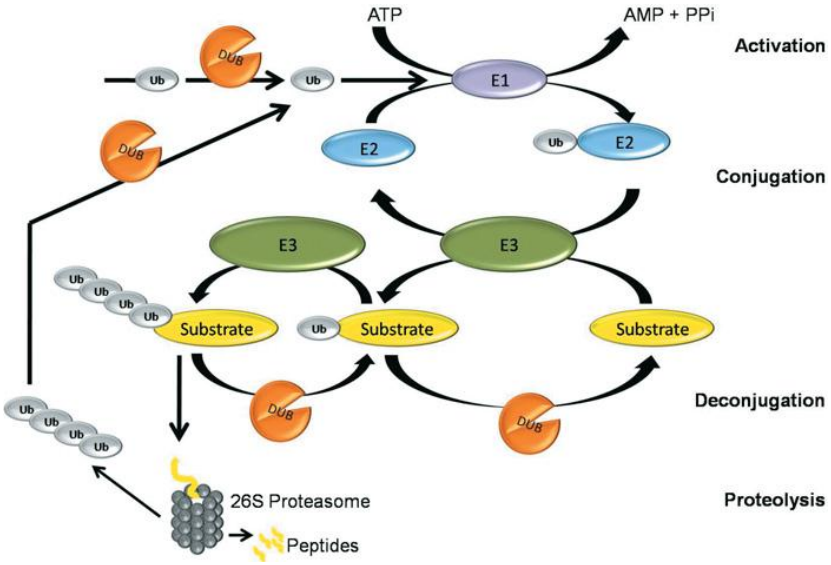

Figure-6: Function of Deubiquitinating Enzymes in Proteolysis. (Ventii & Wilkinson, 2008)

Interestingly, monoubiquitin is more easily removed by DUBs *in vitro* than polyubiquitin chains of four or more, especially those ubiquitins that are linked by lysine 48 (Schaefer & Morgan, 2011), suggesting that proteins with shorter ubiquitin chains are more likely to be rescued from degradation in the proteasome.

DUBs are classified as either cysteine protease DUBs or zinc metalloproteases DUBs (JAMM domain-"JAB1/MPN/MOV34") (Nijman, et al., 2005). The cysteine protease DUBs can be further subdivided into: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), octubain proteases (OTUs), and Machado-Joseph disease proteases (MJDs) (Nijman, et al., 2005). The USPs are the most common type of cysteine proteases, comprising 56 of 79 human DUBs, and 16 of 20 yeast DUBs (called UBPs) (Komander, et al., 2009; Schaefer & Morgan, 2011).

Genetic screens suggest that DUBs perform several functions in the cell. For example, they are involved in synapse function, RNA interference, signaling cascades, gene expression, DNA repair, apoptosis, kinase activation, and other functions (Nijman, et al., 2005; Ramakrishna, et al., 2010). *In vitro*, DUBs have been shown to process ubiquitin precursor proteins, edit ubiquitin modifications by rearranging ubiquitin chains, remove chains from posttranslationally modified proteins, and rescue ubiquitin conjugates (Komander, et al., 2009). Much less is known about DUB functions *in vivo.* DUBs are often associated with other proteins such as substrates, adaptors, and scaffolds, which helps determine their specificity (Sowa, et al., 2009; Kouranti, et al., 2010), however the specific proteins that most DUBs deubiquitinate are largely unknown. Therefore, I have undertaken a study of the 17 highly conserved DUBs in yeast, to develop an approach that can be used to identify *in vivo* targets.

PROJECT PURPOSE

Although a few *in vivo* substrates of DUBs have been identified (Amerik, et al., 2000; Amerik & Hochstrasser, 2004; Nijman, et al., 2005; Hanna, et al., 2006; Komander, et al., 2009), the identities of proteins that yeast DUBs individually target for deubiquitination *in vivo* are largely unknown. It is known, however, that substrate-specific DUBs exist. For example, in human cells USP28 regulates the degradation of the Myc oncoprotein (Popov, et al., 2007). Despite the established connections between DUBs and human disease, no approach has been developed to systematically identify *in vivo* targets of DUBs. My goal is to develop an approach to identify DUB substrates, and thus learn more about DUBs that contribute to human disease.

I hypothesize that overexpression of DUBs that act on ubiquitin-proteasome pathway targets will lead to target protein stabilization, and therefore increased levels of proteasome targets. To test this hypothesis, I will examine the regulation of several Grr1 substrates by yeast DUBs using biochemical and genetic approaches. The biochemical approach will consist of overexpressing each of the known 17 DUBs in yeast, and identifying those that lead to an increase in the stability of any Grr1 targets. This will be done by transforming plasmids encoding each of the 17 DUBs into 2 Grr1 target reporter strains, each expressing three epitope-tagged Grr1 targets. The strains will be grown in galactose to induce transcription and overexpression of the DUBs, and Western blots will be used to analyze the cellular levels of each of the Grr1 target proteins for changes in expression. It is predicted that DUBs that can deubiquitinate Grr1 targets will display an increase in those target's expression upon overexpression *in vivo*.

As an alternate approach, I will look for genetic interactions between DUBs and *GRR1*. Overexpression of *GRR1* leads to slow growth, presumably from accelerated degradation of Grr1 substrates. I will screen for DUBs that reverse this phenotype, by overexpressing both the DUBs

and Grr1 *in vivo* in yeast, and then determining whether any of the DUBs rescue the slow-growth phenotype that is observed following overexpression of Grr1 alone. This will be accomplished by creating strains carrying both a Grr1 overexpression plasmid and one of each of the 17 DUBs. These strains will be serially diluted onto dextrose plates, upon which both Grr1 and DUB expression are silenced, and galactose plates, which will induce the expression of both Grr1 and the DUB. I will then determine if any of the DUBs are able to rescue the slow growing cells. It is predicted that only DUBs that deubiquitinate Grr1 targets will show increased growth on the galactose plates. Together, these approaches will tell us whether we can identify DUB targets *in vivo* by overexpressing individual DUBs. If successful, this approach can be adapted in the future to perform genomic screens to identify all yeast and human DUB targets.

METHODS

Yeast Grr1 Target Reporter Strains

Two yeast strains were created that each had 3 Grr1 targets tagged with different epitope tags, to allow the Western blotting of several different proteins at the same time. To create strain YPS2-4, *CLN2* was tagged with 3HA-KanMX, *CLN3* tagged with 13MYC-HIS3, and *PFK27* was tagged with 3FLAG-Hyg. To create strain YPS5-3, *GIC2* was tagged with 3HA-KanMX, *HOF1* was tagged with 13MYC-HIS3, and *TYE7* was tagged with 3FLAG-Hyg.

Polymerase Chain Reactions

Epitope tag sequences, along with genes encoding selectable markers, were amplified via Polymerase Chain Reaction (PCR) using plasmid DNA templates (pFA6a-3HA-KanMX, p3FLAG-Hygro, pFA6a-13MYC-HIS3MX) and primers that contained sequences to both amplify the appropriate tag and to allow for homologous recombination of the tagging cassette at the desired genomic locus. A sample of each PCR product was then run in a 1% agarose gel with 1 μg/mL ethidium bromide at 140V for 30 minutes, and observed under UV light to make sure the product had been amplified. Once confirmed, the PCR product was ethanol precipitated, and placed in at -20ºC for storage.

High-efficiency Transformation

Prior to transformation, a culture of the parent yeast strain was innoculated in YM-1/2% dextrose, and grown until reaching an OD of 0.5-1.0. The culture was then spun down, washed, and resuspended in Lithium Acetate mix (100 mM lithium acetate, 10 mM Tris-Cl, 1 mM EDTA, pH8). Next, 100 μL of yeast suspension was aliquoted into tubes containing 10 μl of the

PCR product, 100 μg of Salmon Sperm DNA, and 0.7 mL of PEG mix (40% PEG, 100 mM lithium acetate, 10 mM Tris-Cl, 1 mM EDTA, pH8). The samples were incubated for at least 30 minutes at 30ºC. The transformation tubes were then heat shocked for 15 minutes at 42ºC, centrifuged for 30 seconds, and resuspended in 300 μL of YM1/2% dextrose. Finally, the cells were spread on the appropriate selection plates (G418, Hygromycin, -Histidine) and grown at 30ºC.

Zymo Prep Colony PCR

Single colonies that grew on selective plates were transferred using a toothpick onto new selection plates, and checked by PCR to confirm that the tag had recombined into the correct location in the genome. First, genomic DNA was prepared by aliquotting 50 μL of diluted zymolyase enzyme mix into PCR tubes and resuspending single colonies in the tubes. They were incubated for 30 minutes at 37ºC, and then for 10 minutes at 95ºC. The DNA was then aliquoted into new PCR tubes along with the PCR reaction mix, including primers specific to each gene. The PCR program was run, and the product was checked by electrophoresis to see if a PCR product resulted, indicating that the epitope tag sequence had recombined into the correct location.

Yeast GST Fusion Plasmid and Yeast MORF Collection Plasmids

Two different plasmid collections, GST and MORF, were purchased from Open Biosystems. Plasmids in each set include a galactose-inducible promoter that drives expression of each epitope-tagged protein. I utilized 11 GST plasmids and 6 MORF plasmids, each expressing a different DUB gene. All plasmids were confirmed by sequencing before use. The main components of the plasmids and how they are different are illustrated below (**Figure-7**).

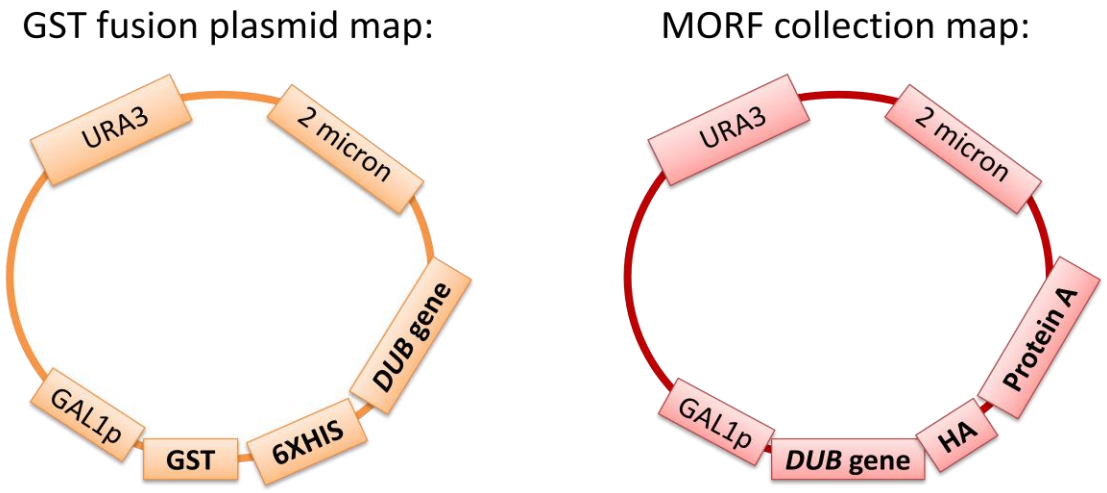

Figure-7: Diagram of GST and MORF Plasmids. The plasmids are similar in that they both include a *URA3* marker, a *GAL1* promoter, and contain a 2 micron origin of replication, meaning that they replicate at a high copy number. They differ because the GST plasmids include both GST and His tags, while the MORF plasmids have HA and Protein A tags.

Plasmid Transformations

Various plasmids were transformed into YPS2-4 and YPS5-3 in order to induce DUB overexpression. Cells were grown to mid-log phase in YM1/2% dextrose media, aliquoted into tubes, centrifuged, and the supernatant was decanted. Next, salmon sperm DNA and the plasmids were added. Then PLATE solution (40% PEG, 0.1 M lithium acetate 10 mM Tris-HCl, pH7.5, 1 mM EDTA) and DMSO were added, and the transformations were incubated at room temperature for at least 30 minutes. They were then heat shocked, centrifuged, and resuspended in TE. Finally, they were plated onto selective plates (-Uracil for *DUB* plasmids, -Leucine for *GRR1* plasmid).

Galactose Inductions

DUB plasmids in YPS2-4 and YPS5-3 were all under the *GAL1* promoter, therefore transcription was induced when galactose was added. Overnight cultures were diluted into 2 identical aliquots of complete medium lacking uracil (to select for cells containing the plasmid) plus 2% raffinose, and grown until cells had doubled. Then, galactose was added to one culture to a final concentration of 2%. The cultures were grown for another 2 hours in 30ºC, and then 6 optical densities (ODs) were collected from each sample. The cells were collected by centrifugation at 4ºC, washed in H_2O, centrifuged again, and pellets were stored at -80ºC.

Western Blotting

Equivalent pellets of cells were lysed in pre-heated SDS sample buffer (50 mM Tris pH7.5, 5 mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, bromophenol blue, 1 μg/mL leupeptin, 1 μg/mL bestatin, 1 mM benzamidine, 1 μg/mL pepstatin A, 17 μg/mL PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate, and 1 mM sodium orthovanadate), and incubated at 95ºC for 5 minutes. Glass beads were then added to samples, bead-beat for 3 minutes in a MiniBeadBeater-96 (Biospec), and then centrifuged for 25 minutes. Extracts were then electrophoresed by SDS-PAGE for 2 hours at 140V, transferred to a nitrocellulose membrane for 2 hours at 0.45 Amps, and western blotted with antibodies against MYC (Clone 9E10, Covance), HA (Clone 16B12), FLAG (Clone M2, Sigma), GST (Clone 4C10, Covance), and Cdc28 (sc-6709, Santa Cruz Biotechnology).

Cycloheximide-Chase Assays

The same protocol as Galactose Induction was used, except after induction, 50 μg/mL cycloheximide was added to cells. Cell pellets from equivalent ODs of cells were collected at 15 minute time points over the course of 1 hour and samples analyzed by Western blotting.

***GRR1* Plasmid Construction**

GRR1 was cloned into a *LEU2*-containing expression vector so that it could be introduced into cells also carrying *URA3*-expressing DUB plasmids. To accomplish this, *GRR1* was amplified from genomic DNA by PCR and cloned into the pRS325-*GAL1p* vector. The cloned plasmid is shown below (**Figure-8**).

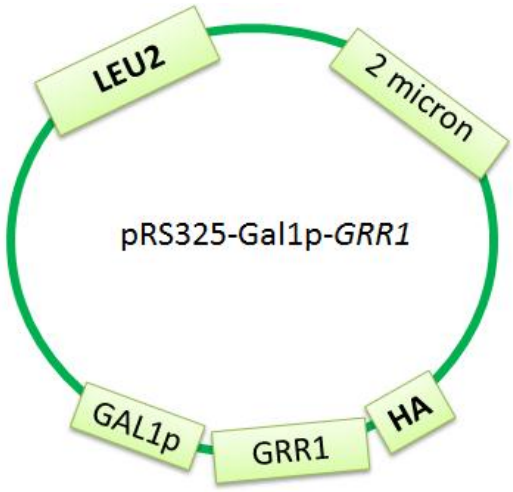

Figure-8: *GRR1* Plasmid Cloning. The plasmid has a *LEU2* marker, an HA tag, a *GAL1* promoter, and contains a 2 micron origin of replication, meaning that it replicates at a high copy number.

Serial Dilution (Spot) Assay

Yeast cultures were innoculated in selective media (C-Uracil, C-Leucine, C-Leucine-Uracil)/2% dextrose, and grown overnight until cells had doubled. Equal aliquots (0.4 ODs)

were centrifuged down, washed twice in media without sugar, and 5-fold serial dilutions were then plated onto dextrose and galactose plates. All plates were incubated at 30°C until control colonies were of equivalent size.

RESULTS

The purpose of this project was to develop biochemical and genetic approaches to identify DUB substrates, to learn more about DUBs that may contribute to human disease.

Initial DUB Tests

Before examining the effect of DUB overexpression on substrates, I sought to confirm that the plasmids expressed each protein of interest, and to screen for potential growth inhibition resulting from overexpression of each DUB. I first sequenced and confirmed 11 GST plasmids and 6 MORF plasmids, each containing 1 of 17 yeast *DUB* genes expressed from the *GAL1* promoter. Three DUBS (UBP8, UBP14, UBP15) were not included in either the GST or MORF collection and were not included in my analysis. The plasmids were transformed into yeast and cells were serially diluted onto dextrose plates (DUB genes off) and galactose plates (DUB genes on) to see whether overexpressing a DUB would cause cell arrest. DUBs *UBP10, UBP3*, and *UBP12* appear to arrest cells on the galactose plate compared to the dextrose plate (**Figure-9**).

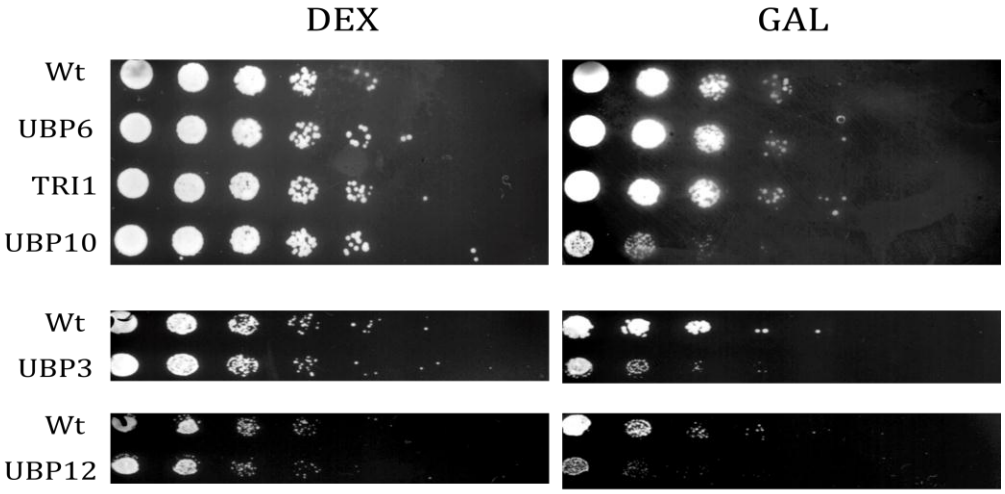

Figure-9: Over-Expression of *UBP10, UBP3*, and *UBP12* Inhibits Growth. Yeast strains containing GST-DUB plasmids were serially diluted 5-fold onto both dextrose (DEX) and galactose (GAL) plates. The plasmids are under a *GAL1* promoter and therefore the *DUB* genes are only expressed when grown on galactose plates.

Next, DUB expression was confirmed in liquid cultures following induction of expression by growth in galactose-containing media (see methods). Western blots were performed to confirm the *DUB* plasmid expression worked. A representative GST blot is shown below (**Figure-10)**.

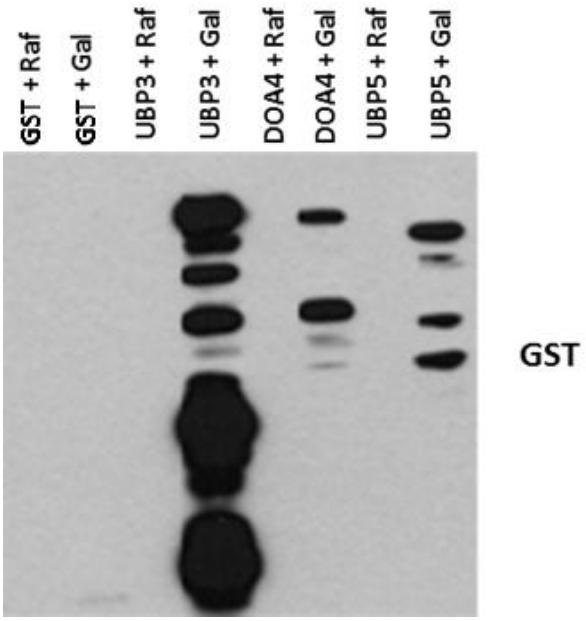

Figure-10: GST Western Blot Confirming DUB Over-expression. Yeast strains carrying the *DUB* overexpression plasmids were grown for 2 hours, and then induced with either raffinose (Raf) or galactose (Gal) for another 2 hours. Samples were then collected, and Western blots were performed with a GST antibody to confirm DUB expression.

Identifying DUBs that Regulate Grr1 Targets

After the all of the *DUB* plasmids had been confirmed for expression and observed for potential cell arrest, they were then analyzed to see whether overexpressing each DUB would increase the stability of any Grr1 target. This was done by first transforming each of the 11 GST plasmids, and an empty vector plasmid, into a target reporter strain YPS2-4 (*MATa his3Δ1 ura3Δ0 leu2Δ0 CLN3-13MYC-HIS3 CLN2-3HA-KanMX PFK27-3FLAG-Hygro*), in which the Grr1 targets Cln2, Cln3, and Pfk27 were tagged. Expression of DUBs was induced by the addition of galactose for 2 hours, samples were collected, and levels of tagged proteins were

assayed by Western blotting. My prediction was that overexpression of DUBs that can deubiquitinate Grr1 targets blocking their degradation would lead to elevated expression of some or all Grr1 targets. Interestingly, I found that *DOA4*, *UBP3*, *UBP5*, *UBP9*, and *UBP12* overexpression each resulted in increased Cln2 protein levels (upper panel) (**Figure-11**). In addition, UBP5 overexpression led to accumulation of a higher molecular weight form of Cln2, potentially representing the phosphorylated protein. However, a second Grr1 substrate, Cln3 (second panel), did not appear to increase following overexpression of any DUB. Laddered bands appear because Cln3 has many phosphorylated forms. Pfk27-FLAG was not detectable in any samples, most likely due to the fact that *PFK27* is not transcribed when cells are grown in galactose (Benanti et al, 2007) (not shown).

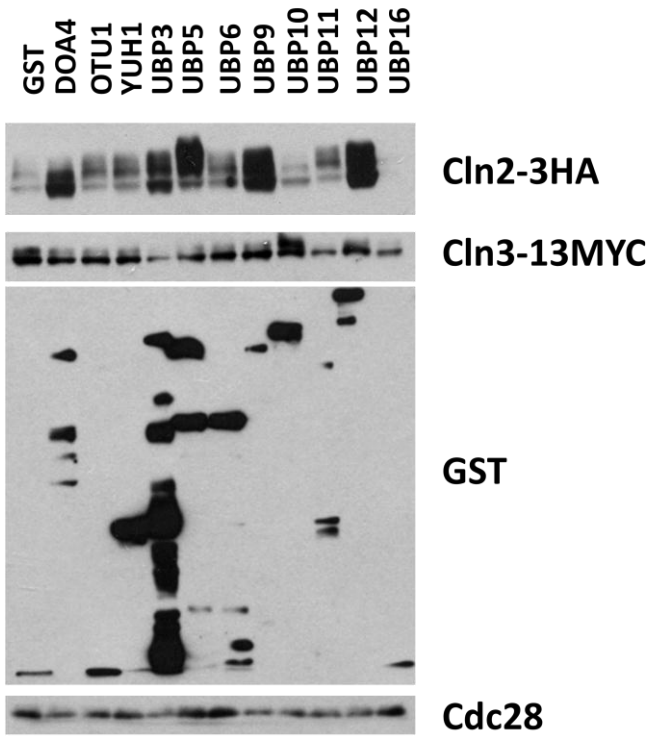

Figure-11: Western Blots of Cln2 and Cln3 in Cells Overexpressing GST-DUBs. GST-DUB plasmids were each transformed into YPS2-4. Galactose was added to the strains for 2 hours allowing for DUB overexpression. Cells were collected and Western blots performed to compare levels of substrate proteins. A GST blot was also performed to verify the plasmids were expressing the DUBs when samples were collected. A Cdc28 blot is shown as a loading control.

Next, the GST plasmids were transformed into the second target reporter strain YPS5-3 (*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 TYE7-3FLAG-Hyg GIC2-3HA-KanMX HOF1-13MYC-His3MX*), in which Gic2, Hof1, and Tye7 were tagged and the experiment carried out as described above. I found that cells over-expressing DUBs *UBP3* and *UBP12* had increased Gic2 substrate protein levels (**Figure-12**), while Hof1 levels did not appear to increase significantly. Similar to the results for Pfk27, Tye7 was not detectable in any sample, consistent with the fact that it is not transcribed when cells are grown in galactose (Benanti et al, 2007) (not shown).

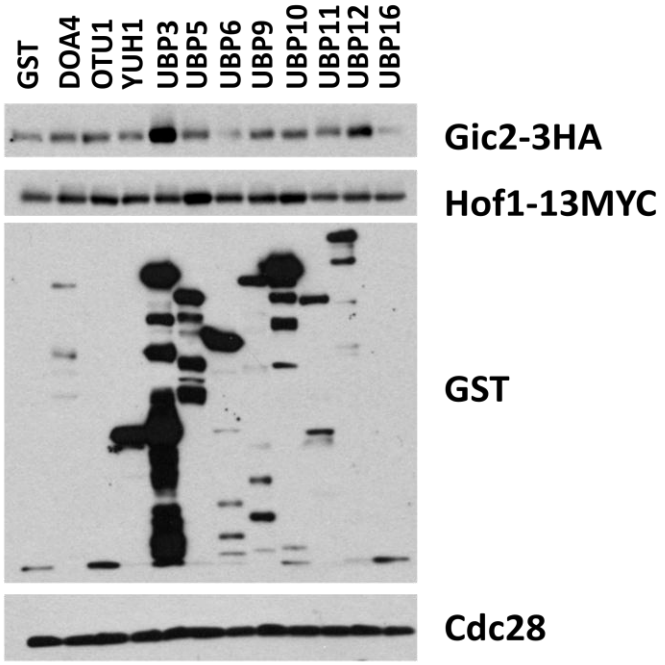

Figure-12: Western Blots of GST-DUBs in YPS5-3 Affecting Gic2 and Hof1 Protein Substrate Levels. GST-*DUB* plasmids were each transformed into YPS5-3. Galactose was added to the strains for 2 hours allowing for DUB overexpression. The samples were collected, and then Western blots were performed for the tagged substrate proteins to observe their levels. A GST blot was also done to verify the plasmids were expressing the DUBs when samples were collected.

Next, the 6 MORF plasmids, along with an empty vector plasmid, were transformed into the same 2 target reporter strains, and the experiments were carried out as before. However, I

found that I could not detect expression of any of the Grr1 targets in these strains because the MORF tag (which contains a Protein A epitope) cross-reacted with all antibodies. For example, an HA blot against Cln2-HA is shown below (**Figure-13**). As seen by the presence of many bands, only the overexpressed MORF-tagged proteins are detectable in the HA-blot. In the future, these DUBs will be cloned into a vector without the MORF tag, so that their effect on Grr1 substrates can be analyzed.

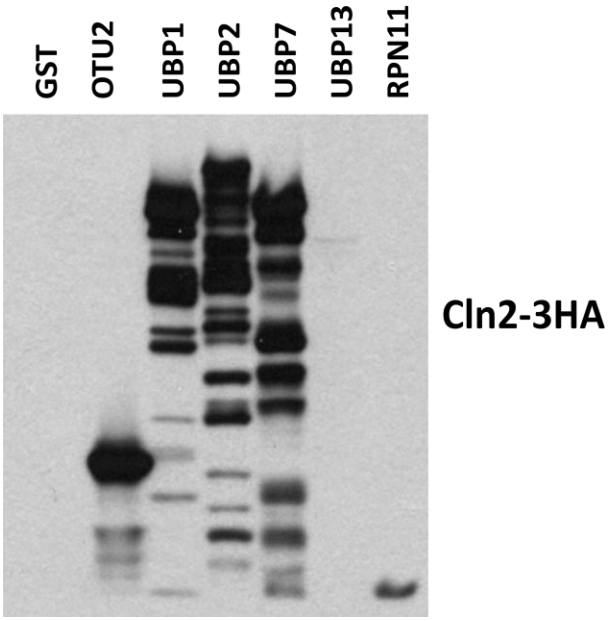

Figure-13: Western Blots of Cross-Reacting MORF-*DUB* Plasmids in YPS2-4. MORF plasmids were each transformed into YPS2-4 and galactose induced for 2 hours allowing for DUB overexpression. Samples were collected, and then an HA Western blot was performed in order to detect Cln2. However, the cross-reactivity of the antibodies with the overexpressed MORF proteins prevented the analysis of Cln2 levels.

My hypothesis is that overexpression of a DUB that can deubiquitinate a particular Grr1 target will lead to increased stability of that target. Since overexpression of 5 GST-DUBs resulted in elevated levels of Cln2, this suggests that these DUBs may stabilize Cln2 or other Grr1 targets. (**Figure-11,-12**). To test this, the half-life of Grr1 targets was analyzed following overexpression of these DUBs. The same process was repeated as before, except cycloheximide

was added to the strains for 1 hour following galactose-induction to inhibit new protein synthesis, and samples were taken every 15 min. Levels of each Grr1 target were then followed by Western blot. I found that *UBP3* and *UBP12* affected Cln2 stability, while *UBP12* affects Cln3 stability (**Figure-14**).

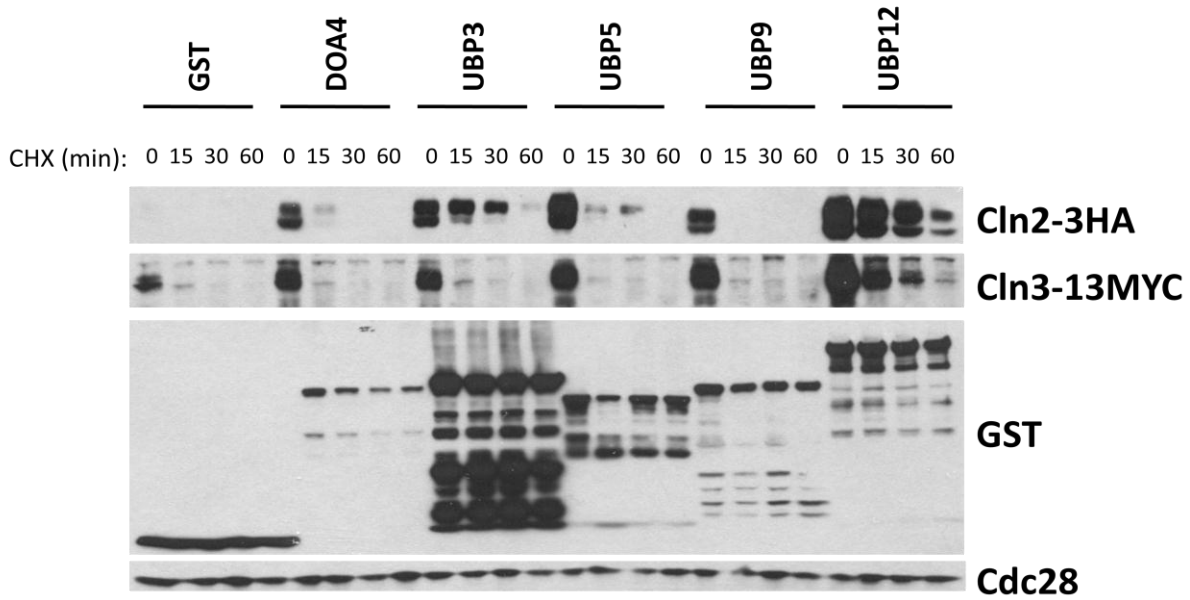

Figure-14: Half-Life Assay of GST Plasmids in YPS2-4. GST-*DUB* plasmids in YPS2-4 were galactose induced for 2 hours allowing for DUB overexpression. Cycloheximide was then added for 1 hour, and 15 minutes samples were taken. Finally, Western blots were performed for tagged proteins in order to observe protein stability over time. A GST blot was also done to verify the plasmids were expressing the DUBs when samples were collected. Experiment was done by B. Landry.

In addition, *UBP3* and *UBP12* affected Gic2 substrate stability (**Figure-15**). Hof1 showed stabilization for all proteins, which is most likely due to the MYC tag (not shown).

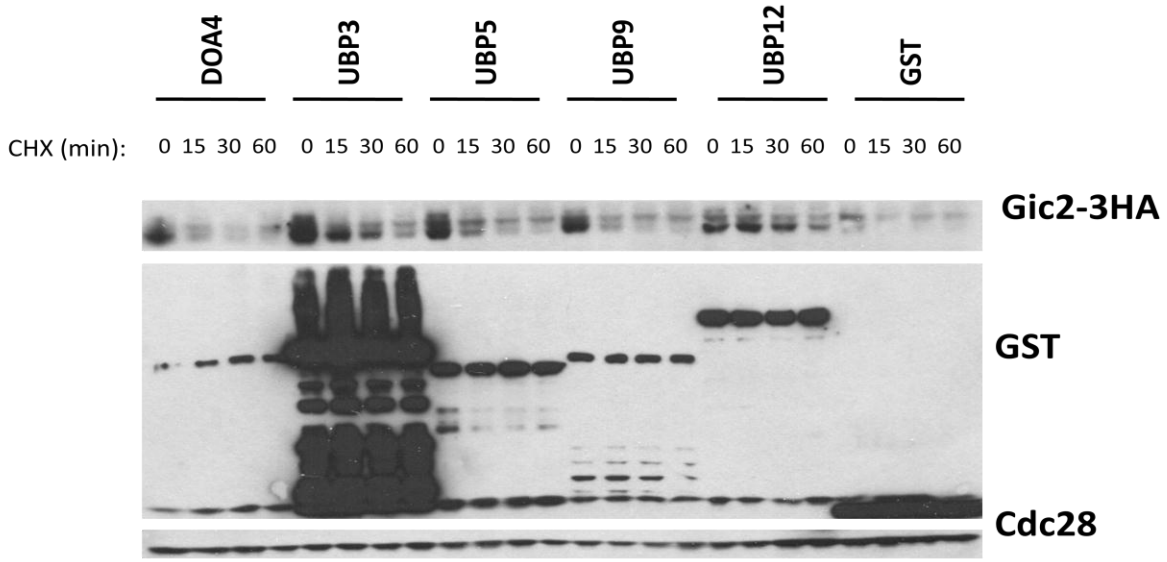

Figure-15: Half-Life Assay of GST Plasmids in YPS5-3. GST-*DUB* plasmids in YPS5-3 were galactose induced for 2 hours allowing for DUB overexpression. Cycloheximide was then added for 1 hour, and 15 min samples were taken. Finally, Western blots were performed for tagged proteins to observe protein stability over time. A GST blot was also done to verify the plasmids were expressing the DUBs when samples were collected. Experiment was done by B. Landry.

Identifying DUBs that Rescue *GRR1* Overexpression

Interestingly, overexpression of *GRR1* has been shown to arrest cells, although the mechanism of this arrest is unknown. One possibility is that overexpression of Grr1 leads to degradation of important substrates, such as G1 cyclins, and cells cannot proceed through the cell cycle without these proteins. A second possibility could be that overexpressing Grr1 leads to saturation of SCF complexes with only Grr1, and therefore no other F-box proteins would be able to bind to the core SCF complex, and therefore the function of these proteins would be inhibited. To test this, I examined the consequence of overexpressing 2 *GRR1* mutants that lack the functional domains of Grr1 (**Figure-16**). Plasmids expressing galactose-inducible full-length Grr1 or each of 2 mutated Grr1 plasmids, 1 with the F-box deleted (Grr1ΔF), so that it cannot bind to the core SCF complex, and 1 with the leucine-rich repeat domain deleted (Grr1ΔL), so that it cannot bind to substrates, were transformed into wild type cells (*MATa his3Δ ura3Δ leu2Δ*

met15Δ). Cells were then spotted onto both dextrose and galactose plates. I found that only overexpression of the full-length Grr1 (figure second row) arrested the cells on galactose plates, meaning a fully functional Grr1 protein that can bind both the SCF complex and substrates is needed to arrest cells. This suggests that the second possibility is not true, because the Grr1ΔL protein can compete with other F-box proteins for binding to the SCF core complex. If the competition model were true, overexpression of Grr1ΔL would be expected to kill cells and it did not. Therefore, Grr1-mediated growth inhibition is likely due to increased degradation of substrates.

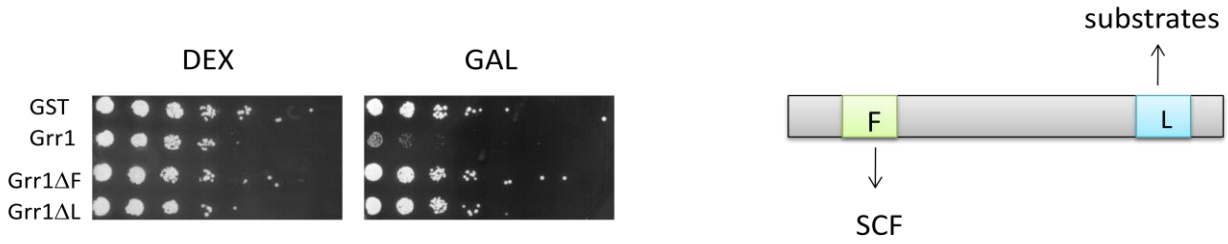

Figure-16: Spot Assay of Grr1 and Mutant Overexpression Plasmids. *GRR1* plasmids (*pYES2-GST-URA3, pYES2-GRR1-URA3, pYES2, GRR1ΔF-URA3, pYES2-GRR1ΔL-URA3*) were transformed into wild type cells, and then serially diluted 5-fold onto both dextrose (DEX) and galactose (GAL) plates. Grr1ΔF represents a plasmid with Grr1 missing the F-box, and Grr1ΔL represents a plasmid with Grr1 missing the LRR domain. The plasmids are under a *GAL1* promoter, therefore the genes are only expressed on GAL plates.

Next, I tested whether overexpression of any DUB could rescue the slow-growth phenotype observed from overexpressing Grr1 alone. This was done by first cloning *GRR1* into a different vector with a *LEU2* marker, instead of a *URA3* marker, since the *DUB* plasmids all carry *URA3* markers and both plasmids required different selectable markers. Then the *GRR1* plasmid and each the 17 *DUB* plasmids (plus a control) were transformed into wild type cells. The strains were then serially diluted onto both dextrose and galactose plates, as explained previously. The prediction was that only DUBs that deubiquitinate Grr1 targets will show restore

normal growth on galactose plates. However, I found that the *LEU2*-containing *GRR1* overexpression plasmid alone (**Figure-17**, second row) did not arrest cells like the *URA3*-containing plasmid had before (**Figure-16**). Therefore, no rescue from DUB overexpression could be determined.

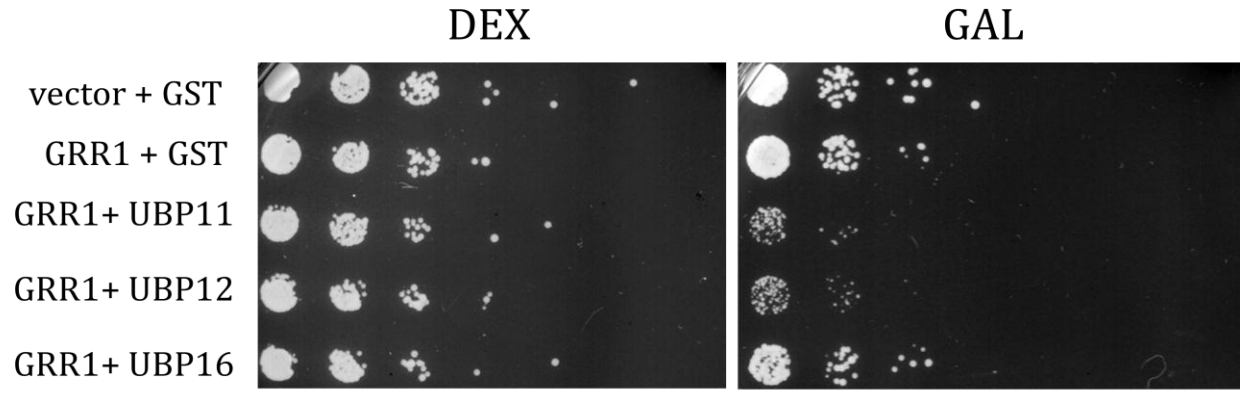

Figure-17: Spot Assay of *GRR1* and DUB Overexpression Plasmids in Wild Type Cells. *pRS325-GRR1-LEU2*, and the indicated DUB plasmids with *URA3* markers, or GST control, were transformed into wild type cells and serially diluted 5-fold onto dextrose (DEX) and galactose (GAL) plates. All genes are transcribed from the *GAL1* promoter, therefore the genes are turned on when on the GAL plates only.

Overall, 17 of the 20 yeast DUBs were analyzed during this project. A list of DUBs tested and the preliminary findings (described below) are summarized in **Table-I**.

DUB Name	DUB Family	Plasmid Type	Expression?	Cell Arrest?	Potential Targets
DOA4	ubiquitin-specific protease	GST	Yes	No	Unknown
OTU1	octubain protease	GST	?	No	Unknown
YUH1	ubiquitin C-terminal hydrolase	GST	Yes	No	Unknown
UBP3	ubiquitin-specific protease	GST	Yes	Yes	**Cln2, Gic2**
UBP5	ubiquitin-specific protease	GST	Yes	No	Unknown
UBP6	ubiquitin-specific protease	GST	Yes	No	Unknown
UBP9	ubiquitin-specific protease	GST	Yes	No	Unknown
UBP10	ubiquitin-specific protease	GST	Yes	Yes	Unknown
UBP11	ubiquitin-specific protease	GST	Yes	No	Unknown
UBP12	ubiquitin-specific protease	GST	Yes	Yes	**Cln2, Cln3, Gic2**
UBP16	ubiquitin-specific protease	GST	?	No	Unknown
OTU2	octubain protease	MORF	Yes	N/A	N/A
UBP1	ubiquitin-specific protease	MORF	Yes	N/A	N/A
UBP2	ubiquitin-specific protease	MORF	Yes	N/A	N/A
UBP7	ubiquitin-specific protease	MORF	Yes	N/A	N/A
UBP13	ubiquitin-specific protease	MORF	Yes	N/A	N/A
RPN11	zinc metalloprotease	MORF	?	N/A	N/A

Table-1: Summary of DUB Analysis. Table 1 shows all the DUBs that were analyzed, the families that they belong to, and the type of plasmid that the *DUB* gene was cloned into. Also shown is whether DUB proteins were expressed from the plasmids, and if the *DUB* plasmids could arrest cells. Potential targets are also shown highlighted in yellow for a few DUBs that have been found based off Western blots shown later in Results. A question mark indicates the protein expression could not be verified, N/A indicates results for the DUB were not tested.

DISCUSSION

This project was successful in identifying candidate yeast DUBs that regulate the degradation of Grr1 targets. Overall, 11 DUBs were analyzed to see if overexpression of any of these could rescue the Grr1 targets Cln2, Cln3, and Gic2 from degradation. Overexpression of six DUBs resulted in increased expression levels of one or more Grr1 targets. These DUBs were analyzed further in half-life assays, and UBP12 overexpression was found to stabilize Cln2, Cln3 and Gic2. In addition, UBP3 overexpression stabilized Cln2 and Gic2.

In vivo Approaches to Identify DUB Targets

It has been shown *in vitro* that DUBs can process ubiquitin precursor proteins, edit ubiquitin modifications by rearranging ubiquitin chains, remove chains from posttranslationally modified proteins, and rescue ubiquitin conjugates (Komander, et al., 2009). However, very little *in vivo* research has been done to understand the roles that deubiquitinating enzymes have preventing protein degradation within the cell and the substrates that they target, even though we know that substrate-specific DUBs exist (Popov, et al., 2007). I took two different approaches, biochemical and genetic, to try and develop an assay that will allow for DUB substrates to be determined *in vivo*.

Biochemical Approach

For the biochemical approach, I overexpressed individual DUB genes and determined if they could regulate the levels of established ubiquitin-proteasome substrates *in vivo*. I focused on proteins targeted by the ubiquitin ligase Grr1 because the structure, function, and targets of Grr1

are well understood (Johnston, 1999) and the pathways regulating the degradation of Grr1 targets have been elucidated. Following DUB overexpression, the stabilities of the tagged Grr1 targets were analyzed by Western blots. DUBs that normally deubiquitinate Grr1 targets were expected to display an increase in those targets' expression upon overexpression *in vivo.* Half-life assays then followed for any DUBs that affected protein stability. In the end this approach was successful in its efforts to identify several candidate DUB substrates.

Genetic Approach

Interestingly, Grr1 overexpression arrests cells, which may be due to accelerated degradation if its targets. If this is true, this phenotype could be used to screen for DUBs that counteract Grr1 function. However, there are two likely possibilities for Grr1 overexpression kills cells: either Grr1 overexpression causes excess substrate degradation (ex. G1 cyclins), or its over-expression leads to saturation of SCF complexes. Using a simple spot assay I was able to find that the former of the two is most likely correct, as a fully functional Grr1 was needed to arrest cells. Therefore, I was interested to know if overexpression of any DUB could prevent this arrest. A newly constructed *GRR1* overexpression plasmid was transformed into wildtype cells along with separate DUB plasmids and then these strains were spotted onto dextrose and galactose plates. DUBs that deubiquitinate Grr1 targets were expected to show increased growth on the galactose plates. However, this newly constructed the *GRR1* plasmid did not arrest cells, so I could not draw any conclusions from these experiments.

Future Directions

Biochemical Approach

Although my results were encouraging, several technical hurdles prevented a comprehensive analysis of all DUBs and Grr1 targets. Since the 6 MORF-DUB plasmids had a Protein A epitope, which binds all immunoglobulins, they cross-reacted with the antibodies that were added to the Western blots, and so target protein levels were not able to be determined (**Figure-11**). These 6 DUB genes will be cloned into the GST plasmids, along with the final 3 DUBs that were missing from the plasmid collections, and the effect of their overexpression on Grr1 target levels will be determined. Second, I originally attempted to examine the Grr1 targets Pfk27 and Tye7, however they were expressed from their endogenous promoters and both promoters are not active in galactose so no protein expression was detected. In the future, we are going to express these genes from a different, constitutive promoter (ex. *TEF1*). Finally, I found that the target Hof1 was stabilized by the addition of the 13MYC tag (data not shown), so Hof1 blots were uninformative.

After assaying all DUBs and Grr1 targets, we will follow-up with half-life assays as we did before using cycloheximide to confirm stabilization over time. In addition, we will perform pull-down experiments to confirm the interaction between DUBs and Grr1 targets.

Genetic Approach

Although I found that only full-length Grr1 could arrest cells upon overexpression, I was not successful in carrying out the DUB screen for technical reasons. The original *GRR1* plasmid that arrested cells contained a *URA3* marker, which was the same marker as the DUB plasmids.

Therefore, *GRR1* had to be recloned into a plasmid with a different marker, *LEU2*, so that both plasmids could be selected for simultaneously. However, the new *GRR1 LEU2* plasmid did not arrest cells like the *URA3* plasmid (**Figure-13,-14**). In the future we are going to make examine differences between the two plasmids that may account for this discrepancy to see if we can confirm the original finding.

Our long term goal is to develop a genome-wide screen that will be able to determine all *in vivo* DUB substrates. My preliminary data suggests that overexpression of DUBs can be used to identify *in vivo* targets. Future experiments will focus on adapting this approach to carry out genome-wide screens.

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